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Molecular Mechanism of the Synaptotagmin-Snare Interaction in Ca^{2+}-Triggered Vesicle Fusion
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SNARE proteins juxtapose synaptic membranes, while certain synaptotagmins are Ca^{2+}-triggers of vesicle fusion in neurons. Despite a wealth of physiological and biochemical data, the molecular mechanism by which SNAREs and synaptotagmin cooperate to link Ca^{2+} and membrane fusion is unknown. The attempts to determine the structure of a synaptotagmin-SNARE complex have been unsuccessful. We combined single molecule fluorescence resonance energy transfer experiments (FRET) and the capture of a SNARE-induced Ca^{2+}-bound state of synaptotagmin (Syt3) by X-ray crystallography. Single molecule FRET showed that the interaction of Syt3 C2AB domains with SNARE complex dramatically amplifies a conformation of Syt3 consistent with crystallographically determined SNARE-induced Ca^{2+}-bound conformation of Syt3. The induction of this Ca^{2+}-bound conformation of Syt3 by the SNARE complex already occurs independent of Ca^{2+}, but it is enhanced in the presence of Ca^{2+}. Remarkably, we found that the spatial arrangement of the two Ca^{2+}-binding regions of this conformation is similar to that of fusion loops of certain viral fusion proteins. Thus, our results reveal the existence of similar structural elements among viral and eukaryotic fusion machineries (transmembrane helices and membrane binding loops) that act in concert to catalyze membrane fusion.

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Selective Exocytosis and Spatiotemporal Regulation of Protein Recycling at the Plasma Membrane
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The concentration of membrane proteins on the cell surface and time they spend in the plasma membrane is defined by dynamic equilibrium between exocytotic delivery and endocytic recycling processes. However, the mechanisms by which cell is able to differentially regulate recycling kinetics of its plasma membrane components is not well understood.
In the current study we present new insights how selective exocytosis and spatial redistribution of proteins in the plasma membrane may regulate their recycling kinetics independent of amount of protein in the plasma membrane. We applied fluorescence photo-activation localization microscopy (FPALM) together with multi-color total-internal reflection fluorescence microscopy to track individual proteins and detect their quantum delivery and internalization via exo- and endocytosis. We showed that upon fusion of exocytic vesicle membrane cargo can be either released into the plasma membrane or retained at the site of fusion. We further provide evidence that protein-specific retention serves as a fusion-associated mechanism for formation of dynamic clusters of proteins in the plasma membrane. These clusters of proteins were found to mediate a dynamic exchange of monomers (protein molecules) with the rest of the plasma membrane and also showed transient association with clathrin. Clathrin assembly at the sites of clusters led to formation of endocytic vesicles that mediated quantum internalization of the clustered protein.
Taken together, these data provide novel mechanism by which spatiotemporal retention and release of membrane proteins during selective exocytosis regulates protein-specific recycling at the plasma membrane. Different models of protein-specific retention/release will be compared and discussed.

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EGFR as a Tool for Probing Productive Endocytosis Events with Epi/TIRF
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Following activation, many receptors at the cell surface partition into clathrin coated pits (CCPs) which subsequently pinch off and move into the cell. Total internal reflection fluorescence (TIRF) microscopy has been used to study such events because it predominantly illuminates <70nm proximal to the coverslip, reducing background fluorescence from the depth of the cell. Loss of clathrin fluorescence from the evanescent field is typically interpreted as endocytosis; however alternative clathrin dynamics at the cell membrane, including formation of clathrin coated pits (CCPs) that are later aborted, has been described. Disappearance of clathrin in TIRF could represent movement of a CCP away from the membrane, uncoating of clathrin from a CCP, or dissociation of clathrin from the cell membrane. To differentiate between these possibilities, we simultaneously imaged a marker which remains with the vesicle. The epidermal growth factor receptor (EGFR) is activated by EGF binding, which induces uptake of its receptor into the cell. In Cos7 cells we analyzed EGFR containing CCPs using Epi/TIR imaging to visualize vesicles as they moved from the cell membrane to deeper in the cell (appearing only in Epi). This allowed us to identify the CCPs that were unambiguously undergoing endocytosis. We demonstrate the utility of EGFR as a model cargo for tracking endocytic vesicles. We tracked individual vesicles following endocytosis, and examined the dynamics of clathrin and EGFR relative to each other. This reveals a range of vesicle behavior as they enter the cell including variable dynamics of clathrin uncoating.

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Real-Time Measurement of Endosomal Acidification by a Novel Genetically Encoded Biosensor
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Genetically encoded fluorescent proteins are optimal reporters when used to monitor cellular processes as they can be targeted to any subcellular region by fusion to a protein of interest. Here, we present the pH-sensitive fluorescent protein E1GFP which is ideally suited to monitor pH changes in dynamic intracellular structures in real time with high spatio temporal resolution. E1GFP is a ratiometric pH indicator by emission with a pK close to 6.0. We describe an application of this novel pH reporter in the measurement of pH changes along the endo-lysosomal pathway. By fusing E1GFP to the HIV-Tat protein which is endowed with cell-penetrating properties, we were able to monitor multi-step endocytosis. It is well established that Tat protein enters T-cells using clathrin-coated pits and progressively translocates into well-characterized low-pH endosomes. By using E1GFP conjugated to Tat we monitored the changes in vesicle pH, starting from the initial cell-surface value through the intracellular endocytic network, in real time. We demonstrate a progressive acidification within Tat-loaded vesicles that is crucial to the intracellular fate of Tat. The low endosomal pH exploited by Tat-E1GFP after prolonged incubation in live cells, leads to protein degradation in the lysosomal compartment. We also show that treatment with chloroquine raises the vacuolar pH, demonstrating the ability of E1GFP to respond effectively to environmental pH changes. We believe that this implementation illustrates the suitability of E1GFP for use in the surveillance of intracellular pH during dynamic cellular events with high spatio-temporal resolution.

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Integrative Experimental and Theoretical Approach Exposes Fundamental Mechanisms of J774 Macrophage Phagocytosis
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Macrophage cell lines like murine J774 cells are ideal model systems to study phagocytosis. Our unique methodology combines single-cell/single-target experiments with advanced computer simulations to elucidate the fundamental mechanisms of J774 macrophage phagocytosis. As a first step, the baseline mechanical properties of the murine macrophages were established. Micropipette-aspiration experiments were used to characterize the cortical tension and cytoplasmic viscosity of the J774 cells. Next, we used a dual-micropipette manipulation system to quantify the time courses of a number of key parameters during Fcγ-target phagocytosis. A passive cell is selected and picked up with a micropipette by partial aspiration. Another micropipette is used to bring an opsonized target bead (5-30 um diameter) into soft contact with the cell, which usually results in immediate adhesion. The target is released and images of the ensuing phagocytosis are recorded directly to a computer hard disk. Their analysis provides the time course of cell morphology, bead position, and cortical tension. Intriguingly, the macrophages maintained a constant cortical tension when engulfing targets that required a surface area expansion of up to ~250%, indicating an extremely large membrane reservoir. The tension rose when the cells increased their surface area by an amazing ~550%. These and other experimental observations were compared side-by-side with finite-element models of macrophage phagocytosis. This comparison allows us to assess the viability of different mechanistic models used for phagocytosis. Optimal models include a repulsion between the cytoskeleton and the free membrane (which drives protrusion), and an attraction between cytoskeleton and surface membrane newly adherent to the target (which results in thin pseudopods). Confocal inspection of the engulfment of 20 um beads by GFP-actin transfected macrophages revealed excellent agreement between the cytoskeletal density predicted by the optimal model and the observed actin distribution.